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ABSTRACT

Pregnancy specific glycoproteins (PSGs) are secreted proteins of unknown function that belong to the carcinoembryonic antigen gene family (CEA). CEA is a commonly used tumor marker for adenocarcinomas. Expression of PSGs in normal breast tissue is undetectable by current techniques but they have been shown to be expressed in certain tumors including breast ductal and lobular carcinomas by immunohystochemistry. Because polyclonal anti-PSG antibodies can cross react with other members of the CEA family, we have examined the expression of PSGs by reverse transcriptase-polymerase chain reaction with PSG-specific primers. Our results indicate that all 8 breast cancer cell lines expressed mRNA encoding for PSGs and their splice variants. When breast tumors were examined, 29 out of 89 tumors expressed PSGs. When we looked at the different PSGs expressed in these tumors, we found that they all expressed PSG 4 and some other PSG. Several splice variants were detected some of which may have been previously undetected in placental tissue. We have produced one recombinant PSG containing the integrin binding motif, RGD, and one lacking the RGD tripeptide in insect cells. These proteins were produced as fusions with glutathione-S-transferase and have the predicted molecular mass. These proteins were used in studies to determine if they have any effect in cell proliferation of a breast cancer line. No effects in cell proliferation were detected and our binding studies indicated that they most likely do not express receptors in the cells that produce them.

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INTRODUCTION

Pregnancy specific glycoproteins (PSGs) are a subfamily of secreted proteins within the carcinoembryonic antigen family(1). Due to their sequence and domain structure, PSGs have been included in the immunoglobulin gene superfamily. In humans, PSGs are derived from 11 closely related genes organized in tandem in chromosome 19. In addition to the 11 different mRNAs that are encoded by the 11 PSG genes, the number of PSG mRNAs is increased due to alternative splicing and the use of alternative polyadenylation signals (2). Alternative splicing generates proteins with different number of domains from the same mRNA. They have been classified as type I (three immunoglobulin-like constant domains) and type II (two Ig-like domains)(5). All splice variants conserve the leader peptide and therefore these proteins are all secreted from the cell. Some PSGs such as PGS 11, have the tripeptide sequence Arg-Gly-Asp (RGD) in the N-domain while others such as PSG 1do not. The RGD sequence is present in a variety of extracellular matrix proteins that bind to integrin receptors such as fibronectin and vitronectin. The extracellular matrix regulates many aspects of the cell phenotype including differentiation state, control of gene expression, apoptosis, and cell proliferation. In mammary epithelial cells, the basement membrane has a central role in controlling gene expression and a signal transduction cascade involving integrin receptors mediates this process. PSGs have also been cloned in other species including primates, rats, and mice but the function of PSGs remains unknown.

Normal breast tissue does not express PSGs as indicated by the lack of amplification of PSG cDNAs by reverse transcriptase polymerase chain reaction (RT-PCR)(1). Our group confirmed this observation. Elevated levels of PSGs have been reported for some tumors including choriocarcinomas, chorioepiteliona, invasive moles, and breast carcinomas. Published reports vary substantially in the accounted percentage of breast tumors that present with elevated PSG levels. The discrepancies can be accounted for by different factors: the anti-PSG antibodies used for the study, the fixation technique employed, the sensitivity of the technique, and the difference in the secretory capacity between tumors when serum levels were measured. In addition, PSGs are highly homologous at the amino acid level and the antibodies employed in the studies could not discriminate between the different members of the PSG family. Therefore unless the study included analysis of the PSG mRNAs by RT-PCR with primers specific for each PSG, no information related to which PSGs are overexpressed is available.

We proposed to:

1. Analyze pregnancy specific glycoprotein mRNA expression in human breast cancer lines and breast cancer tissue.
2. Transfect the selected PSG cDNAs in breast cancer lines and establish stably transfected cell lines.
3. Obtain large quantities of purified PSGs for future functional studies.

PROGRESS REPORT
Results obtained from 7/97 to 8/98

Analysis of PSG mRNA expression in human breast cancer lines:
During the first year of this proposal we analyzed a series of breast cancer cell lines for expression of PSG mRNAs. These included cell lines that are estrogen receptor positive, estrogen receptor negative, a cell line that is derived from normal marginal breast tissue (Hs578 Bst), and a cell line derived from a spontaneously immortalized breast epithelial line (MCF 10a). We found that all the cell lines tested expressed PSGs although in some cell lines the level of expression was low. To the eight cell lines tested in the first year, we added three more lines: BT-20, ZR-75-30, and SK-BR-3.We found that by RT-PCR these three cell lines expressed PSG mRNAs.

Analysis of pregnancy specific glycoprotein mRNA expression in breast cancer tissue:

We analyzed the expression of PSGs using specific primers for PSG 1, 4, 5, and 11. The tumors used for this study were selected after an analysis of the expression of PSGs with a primer set that does not distinguish between the different members of the family and therefore was called the primer set PSG uni. We only analyzed for specific expression of PSGs, the tumors that were positive for amplification with the PSG uni set (18 out of 81) and numbered the samples from 1 to 18.

Amplification of PSGs from Invasive Ductal Carcinomas

Tissue Sample #	**PSG #1**	**PSG #4**	**PSG #5**	**PSG #6**	**PSG #11**
1	+	+	+	+	+
2*	-	+	+	+	+
3	-	+	+	+	+
4**	+	+	+	-	+
5	+	+	-	+	-
6	+	+	+	+	-
7	+	+	+	+	+
8	+	+	+	-	+
9	-	-	+	-	-
10	-	+	-	+	-
11	-	+	+	+	+
12	-	+	-	-	+
13	-	+	-	-	-
14	+	+	+	-	-
15	-	+	-	+	+

16	+	+	-	+	+
17	+	+	-	-	+
18	+	+	+	-	+

*Not certain if ductal or lobular breast tumor
**Invasive lobular carcinoma

We also analyzed mRNA extracted from 17 paraffin embedded breast tumors according to a published protocol. To determine the quality of the extracted RNA, we first amplified it with primers for the housekeeping gene GAPDH. We obtained RNA that was not degraded and had no enzyme inhibitors from only 8 of the 17 samples. Of these 8 samples, expressed PSGs, two were derived from lobular and three from ductal breast carcinomas.

<u>Effects of PSG 1 and PSG 11 in proliferation of Hs578T cells:</u>
Because we could not find a breast cancer derived cell line that does not encode for PSGs, we decided to modify the second specific aim of our original proposal as suggested in the first progress report. Our third proposed objective was to obtain large quantities of purified PSGs. At the end of the first year of this proposal, we had been able to produce two PSGs, PSG 1 and PSG 11 in a baculovirus expression system. We picked these two PSGs based on the fact that PSG 1 does not contain the RGD sequence in its N-domain while PSG 11 does. RNA encoding for these two PSGs was detected in breast cancer lines as well as breast tumors (see table above). The proteins were produced as fusions with the GST tag and we were able to show that they are glycosylated and react with both anti-GST antibodies and anti-PSG monoclonal antibodies. We also generated a GST fusion protein, GST-XylE to use as control for the experiments. We scaled up the production and purification of these three proteins and tested their effect on growth of the breast ductal carcinoma line Hs578T. For this purpose, we used a cell proliferation kit from Amersham that measures cell proliferation by detecting incorporation of bromodeoxyuridine (BrdU) into cellular DNA with an anti-BrdU monoclonal antibody. Four different treatments were included in the experiment and each treatment was done in three wells each seeded with 1×10^4 cells in a 100 μl volume. The treatments included 5 μg/ml of GST-PSG 1, GST-PSG 11, GST-XylE (control) or media alone. Cells were incubated with the indicated proteins for 72 hours and cell growth was determined using an ELISA reader that measures the signal produced by binding of the anti-BrdU antibody. We saw no significant difference between all four treatments, which indicates that PSG 1 and PSG 11 have no effect on proliferation of the cell line tested.

<u>Binding of PSGs to breast cancer lines:</u>
To determine if PSGs produced by the breast cancer lines bind back to the cell that produced them, we designed binding assays using PSG-placental alkaline phosphatase (PSG-AP) fusion proteins. Dr. Flanagan (Harvard University) first described the binding assays using AP-fusion proteins and we obtained the plasmids necessary to generate the PSG 1d-AP as well as the plasmid AP-Tag 4 from him (11). The AP-Tag 4 plasmid produces AP protein that is secreted into the supernatant upon transfection into

eukaryotic cells and is used as the control for the binding assay. We transfected the plasmid containing the cDNA for AP-PSG1d and AP only into 293T cells using the lipofectamine plus reagent (LTI). An antibody to the AP protein is commercially available but does not react with the denatured protein. Therefore, we have used the anti-AP antibody to immunoprecipitate the AP and the AP-PSG 1d proteins from the media of transiently transfected cells. We found that the highest level of recovery of these proteins was at 5 days post-transfection. Analysis of the immunoprecipitated material showed that a protein of 67 kDa, corresponding to the AP, and a protein of approximately 130 kDa, corresponding to the estimated size of the AP-PSG 1d fusion protein, were present. Furthermore, the AP-PSG1d fusion protein reacted with the BAP-1 monoclonal anti-PSG antibody in Western blots. We estimated the amount of AP and AP-PSG 1d protein by comparison of the immunoprecipitated material to BSA standards run in the same gel after staining with the Gel Code Commassie blue reagent (Pierce). The concentration of protein obtained by this method was confirmed by the method of Flanagan and Leader that is based on the colorimetric changes of the substrate for AP, p-nitrophenyl phosphate. For the binding assays, 5X 10^5 Hs578T cells per well were seeded in 24-well plates. To each well, increasing concentrations of AP or AP-PSG1d were added and cells were incubated for 90 min on ice. The cells were then lysed and endogenous cellular AP activity was inactivated by incubation of the lysate at 65 degrees for 10 min. Bound AP was estimated after addition of the AP substrate for an hour in an ELISA reader. Values obtained upon incubation of the cells using AP were compared to values obtained using the AP-PSG 1d protein. We did not observe a significant difference in binding between the AP and the AP-PSG 1d assay to the breast cancer line when low protein amounts were used for the assay. At higher concentrations of protein, we observed a slight increase in binding of AP-PSG1d over the binding of AP only. Recently, we have performed the same assay using monocytic cell lines. Our laboratory has found that murine PSGs bind to macrophages and induce production of IL-10 (Dveksler, in preparation). Therefore, as a positive control we used a monocytic cell line and repeated the binding assay. We found a difference in binding between the AP and AP-PSG fusions at both a low and a high protein concentration. Therefore, we believe that the small difference in binding detected for the Hs578T cells could represent a very low affinity receptor for PSG or increased background binding of the PSG1d-AP over the AP only. This increased background or "non-specific stickiness" could be due to the high degree of glycosylation present in the PSG-1d.

CONCLUSIONS

We have determined that mRNA encoding for all human PSGs is expressed in a variety of human breast-derived cell lines including: MCF-7, T47D, ZR-75-1, Hs578T, BT 747, BT 483, MCF 10a, Hs578Bst, BT-20, ZR-75-30, and SK-BR-3. These cells include estrogen receptor negative and estrogen receptor positive cells. We have also confirmed that some human breast tumors express PSGs while others do not. Normal breast tissue does not express PSGs. When we analyze which PSGs were expressed in the tumors where PSG mRNA was detected, we found that different tumors expressed different

PSGs and that more than one splice variant per PSG could be detected. Most tumors expressed PSG 4 and PSG 1, 5, 6, and 7 were expressed in some but not all tumors. It is important to note however, that in some instances, we detected a PCR product for some of these PSGs that hybridized to the specific probe but was of the incorrect size. In the table showed above, we indicated that there was no expression of the specific PSG but we can not exclude the possibility that a different splice variant which results in a PCR product of a different size not previously identified by us or others is expressed in these tumors. We had great difficulty extracting RNA compatible with RT-PCR amplification from paraffin-embedded tissue samples that we obtained from NNMC. Of the 17 different tissue blocks we obtained, we could only prepare RNA of enough quality for analysis by RT-PCR from 8 of them. Although we followed published protocols, we noticed that the time employed during the fixation, the age of the blocks, and the efforts to attempt to do the sample handling prior to fixation in an RNase free-environment were essential to the success of the procedure (4). Unfortunately, we could not control any of these parameters because we were only allowed to use archived material and therefore we did most of our studies using all the samples that could be sent to us by our collaborator, Dr. Thompson in Germany. Dr. Thompson had access to invasive carcinomas and was able to freeze the tissue for RNA extraction in liquid nitrogen immediately upon resection.

Our laboratory has recently determined that members of the murine PSG family induce IL-10 in peritoneal exudate macrophages and a macrophage cell line (6). Macrophages do not express PSGs but are known to infiltrate tumor sites. Richter et al., reported that expression of IL-10 in tumor cells resulted in a loss of tumorigenicity. They hypothesized that IL-10 could indirectly suppress tumor growth of certain tumors by inhibiting infiltration of macrophages, which provide tumor growth promoting activity. Kundu and co-workers have shown that IL-10 has antimetastatic and antitumor activity in a murine mammary tumor model (7). The authors found that this activity of IL-10 is related to enhanced production of nitric oxide and its ability to downregulate class I expression, leading to enhanced NK lysis of tumor cells (8). Interestingly, IL-10 has been suggested to play several roles in oncogenesis, from stimulating tumor growth and metastasis to enhancing tumor regression. There is not enough information to predict whether IL-10 has a positive or negative role in neoplasia and part of the controversy is probably related to the fact that its activity will depend on the tumor cell type (9, 10).

Our studies using two members of the human PSG family, PSG 1d (non RGD-containing PSG) and the PSG 11 (RGD-containing PSG) indicate that PSGs do not have growth promoting activity in the breast cancer line tested. Furthermore, binding studies with AP-PSG 1d suggest that PSG1d most likely does not bind to this cell line. Due to our recent discovery that PSGs induce IL-10 in macrophages, it can not be ruled out that tumors expressing PSGs may have an effect in regulating the immune environment and this in turn, may influence the patient's immune system ability to fight the tumor. Studies that investigate the role of IL-10 in neoplasia are underway in many laboratories and it is anticipated that given its possible pleiotropic roles, both IL-10 and its antagonists will be therapeutically important.

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David Wessner, Ph.D.

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